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Colonization of Transgenic Tobacco Constitutively Expressing Pathogenesis-Related Proteins by the Vesicular-Arbuscular Mycorrhizal Fungus *Glomus mosseae*

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We studied the effect of constitutive expression of pathogenesis-related proteins (PRs) in tobacco plants on vesicular-arbuscular mycorrhiza. Tobacco lines genetically transformed to express various PRs constitutively under the control of the cauliflower mosaic virus 35S promoter of tobacco were examined. Immunoblot analysis and activity measurements demonstrated high levels of expression of the PRs in the root systems of the plants. Constitutive expression of the following acidic isoforms of tobacco PRs did not affect the time course or the final level of colonization by the vesicular-arbuscular mycorrhizal fungus *Glomus mosseae*: PR-1a, PR-3 (=PR-Q), PR-Q′, PR-4, and PR-5. Similarly, constitutive expression of an acidic cucumber chitinase, of a basic tobacco chitinase with and without its vacuolar targeting peptide, of a basic β-1,3-glucanase, and of combinations of PR-Q and PR-Q′ or basic chitinase and basic β-1,3-glucanase did not affect colonization by the mycorrhizal fungus. A delay of colonization by *G. mosseae* was observed in tobacco plants constitutively expressing the acidic isoform of tobacco PR-2, a protein with β-1,3-glucanase activity.

Plants react to infection with necrotizing pathogens with an array of biochemical reactions collectively called the defense response; an important part of this response is the accumulation of the so-called pathogenesis-related proteins (PRs) (10, 17, 34). In tobacco plants, mRNAs for all these PRs accumulate coordinately and systemically, and the expression of the PR genes correlates with the induction of systemic acquired resistance against various pathogens (28, 39). Among the PRs are chitinases and β-1,3-glucanases, enzymes which degrade fungal cell walls and inhibit fungal growth (2, 3, 19, 20), and it is tempting to speculate that these PRs are causally involved in disease resistance (28, 39). Plants constitutively expressing individual PRs were used to test this hypothesis (28). Transgenic plants expressing constitutively high levels of chitinase showed higher levels of resistance to the root pathogen *Rhizoctonia solani* (8, 36) but not to the leaf pathogen *Cercospora nicotianae* (22). Constitutive high-level expression of PR-1a in transgenic tobacco resulted in increased tolerance to two oomycete pathogens, *Peronospora tabacina* and *Phytophthora parasitica* (1). Furthermore, constitutive coexpression of chitinase and β-1,3-glucanase led to protection of tobacco against *C. nicotianae* (42). Thus, expression of PRs may be a strategy for crop protection (9, 15).

Most herbaceous plants are hosts for vesicular-arbuscular mycorrhizal (VAM) fungi that enhance the uptake of mineral nutrients in exchange for assimilates provided by the plant (13, 31). Colonization by VAM fungi is, in many ways, comparable to an infection by pathogenic fungi, and it is interesting to examine how VAM is affected in plants expressing PRs for enhanced pathogen resistance. We previously showed that *Nicotiana sylvestris* plants expressing high levels of chitinase were colonized to the same extent as control plants with the VAM fungus *Glomus mosseae* (36). In this study, we extended such tests to transgenic tobacco plants constitutively expressing high levels of different PRs.

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MATERIALS AND METHODS

Plant material. Homozygous tobacco (*Nicotiana tabacum* cv. Xanthi NC) transformants selected to express high levels of different transgenes were used in this work (Table 1). They had been obtained by *Agrobacterium*-mediated leaf disk transformation with derivatives of the binary vector pCGN1774, which contained inserts with chimeric genes encoding the desired protein under the control of a cauliflower mosaic virus 35S promoter (1). Wild-type tobacco plants and tobacco plants transformed with the same vector without inserts ("empty-vector plants") were used as controls. Seeds for all plants were kindly provided by J. Ryals (Ciba, Research Triangle Park, N.C.); the plants were grown in a greenhouse (day-night cycle: 14 h at 27°C for the day portion and 10 h at 20°C for the night portion) in a steam-sterilized (40 min at 121°C) mixture of sand and loam (1:1 vol/vol).

Analysis of establishment of VAM. To analyze colonization by the VAM fungus *G. mosseae*, a previously described container system was used (41). Soybean (*Glycine max*) seedlings (6 days old) were planted into the inoculum-plant container, which was equipped with nylon screens (60-μm mesh size) as side walls; an inoculum of *Tagetes tenuifolia* roots colonized with *G. mosseae* was used (36).

Tobacco plants were first grown for 4 weeks in a sterilized nutrient-rich substrate and then transplanted into the steam-sterilized sand-loam mixture in a series of test plant containers similarly equipped with side walls of nylon screen (41). These side containers were inclined at a 45° angle so that roots grew along the nylon screen. After a further period of 2 weeks, the test plant containers were joined with the inoculum container so that the two nylon screens were in intimate contact.

Test plants were harvested 2 to 8 weeks after the containers were joined. Roots were harvested, cleared, and stained as described previously (27), and the level of colonization by the VAM fungus was estimated by the grid line intersect method under a dissecting microscope (12). Data are given as percentages of root length colonized. Where required, a sample of root pieces was randomly picked (total length, 5 cm), and the lengths of the areas containing VAM structures were measured under the microscope.

The experiments were performed twice, with at least five replicates per transgenic line in each experiment. Similar results were obtained in both experiments. Means and standard errors of the means of the second experiment are given.

Enzyme assays. Plant material was ground in a mortar on ice with a pestle in homogenization buffer (50 mM Tris, adjusted with HCl to pH 8.0, containing 500

TABLE 1. Transgenes expressed in the tobacco plants used in the present study

Transgene	Characteristics of the encoded protein[a]	Reference
PR-1a	Acidic, extracellular; function unknown	1
PR-2	Acidic, extracellular β-1,3-glucanase of class II	38
PR-3 (=PR-Q)	Acidic, extracellular chitinase of class II	24
PR-4	Acidic, extracellular; function unknown	11
PR-5	Acidic, extracellular; function unknown	25
PR-Q′	Acidic, extracellular β-1,3-glucanase of class III	26
Basic chitinase	Basic, vacuolar chitinase of class I	22
Basic chitinase ΔVTP[b]	Basic chitinase of class I lacking the C-terminal vacuolar targeting peptide	23
Basic β-1,3-glucanase	Basic, vacuolar β-1,3-glucanase of class I	30
Cucumber chitinase	Acidic, extracellular chitinase of class III	21

[a] More detailed information can be found in recent reviews (10, 17, 20).
[b] VTP, vacuolar targeting peptide.

mM NaCl and 0.2% Triton X-100) and centrifuged at 10,000 × *g* for 15 min. Chitinase in the supernatant was assayed as described previously with [^{3}H]chitin as a substrate (4). The activity of β-1,3-glucanase was assayed with laminarin as a substrate (18), using extracts dialyzed for 13 h against 2.5-fold-diluted homogenization buffer. Activities were expressed on a protein basis; the protein concentration was determined according to the method of Bradford (7) with bovine serum albumin as a standard.

Expression of PRs. The expression of PR-1a, PR-2, PR-3 (=PR-Q), PR-4, PR-5, and PR-Q′ was tested on immune blots (33) after separation of the proteins on sodium dodecyl sulfate (SDS)–10% polyacrylamide gel electrophoresis (PAGE) gels (14). The polyclonal antisera, raised in rabbits (dilutions: PR-1a, 1:100; PR-2, 1:1,000; PR-3 [=PR-Q], 1:2,000; PR-4, 1:500; PR-5, 1:1,000; and PR-Q′, 1:2,000), were kindly provided by Tom Luntz (Ciba, Research Triangle Park, N.C.).

RESULTS

Expression of PRs in the roots of transgenic plants. The roots of transgenic tobacco lines expressing basic chitinase, basic chitinase lacking the C-terminal vacuolar targeting peptide, or basic chitinase in combination with basic β-1,3-glucanase showed high levels of chitinase activity (Table 2). In the transformants expressing the cucumber chitinase, the total chitinase activity in the roots was increased only slightly. Enhanced β-1,3-glucanase activities could be observed in the transformants expressing basic β-1,3-glucanase alone as well as in combination with basic chitinase (Table 2).

TABLE 2. Enzyme activities in roots of untransformed *N. tabacum* plants and in plants expressing enzymes[a]

Transgene expressed	Sp act in roots[b] (nkat/mg of protein)	
	Chitinase	β-1,3-Glucanase
None (untransformed plant)	2 ± 1.3	16 ± 5.9
Empty vector	5 ± 2.7	24 ± 4.1
Basic chitinase	280 ± 22	ND[c]
Basic chitinase ΔVTP[d]	78 ± 11	ND
Cucumber chitinase	13 ± 4	ND
PR-3 (=PR-Q)	8 ± 1.9	ND
Basic β-1,3-glucanase	ND	50 ± 7.4
PR-Q′	ND	25 ± 4.8
PR-2	ND	23 ± 5.0
PR-3 + PR-Q′	6 ± 2.1	23 ± 2.8
Basic chitinase + basic β-1,3-glucanase	211 ± 28	53 ± 3.2

[a] *N. tabacum* lines were untransformed plants or homozygous progeny from plants transformed with a vector containing no inserts (empty vector) or chimeric genes encoding different proteins; the plants expressed various forms of chitinase or β-1,3-glucanase under the control of a cauliflower mosaic virus 35S promoter.
[b] The specific activities of chitinase and β-1,3-glucanase were measured 6 weeks after plants were transferred into an autoclaved sand-loam mixture.
[c] ND, not determined.
[d] VTP, vacuolar targeting peptide.

TABLE 3. Colonization of transgenic tobacco plants expressing various PRs

Transgene expressed	% Colonization[a] by *G. mosseae* after:			
	2 wk	4 wk	6 wk	8 wk
None (untransformed plant)	2.0 ± 1.2	12 ± 3.0	23 ± 3.9	40 ± 4.5
Empty vector	2.3 ± 0.3	14 ± 2.4	22 ± 4.4	43 ± 5.6
PR-1a	2.7 ± 0.9	8 ± 3.5	21 ± 1.6	44 ± 1.6
PR-2	1.9 ± 0.6	1 ± 1.0	8 ± 5.0	24 ± 5.0
PR-3 (=PR-Q)	3.7 ± 1.8	11 ± 0.9	20 ± 4.1	44 ± 5.1
PR-4	2.3 ± 0.7	10 ± 2.1	18 ± 5.4	40 ± 4.2
PR-5	3.3 ± 1.9	12 ± 3.3	20 ± 7.1	35 ± 7.2
Basic chitinase	2.1 ± 0.6	11 ± 1.7	17 ± 3.9	40 ± 8.1
Basic chitinase ΔVTP[b]	1.7 ± 0.9	11 ± 1.9	21 ± 2.0	38 ± 6.1
Cucumber chitinase	2.3 ± 0.3	11 ± 2.0	19 ± 3.9	37 ± 5.4
Basic β-1,3-glucanase	2.0 ± 1.0	10 ± 1.2	19 ± 2.3	39 ± 5.4
PR-Q′	2.2 ± 0.6	14 ± 3.2	18 ± 6.5	49 ± 7.5
PR-3 + PR-Q′	1.9 ± 0.6	12 ± 4.0	21 ± 4.7	44 ± 6.7
Basic chitinase + basic β-1,3-glucanase	3.7 ± 0.3	15 ± 3.6	23 ± 3.4	38 ± 4.6

[a] Each value is the mean and standard error of the mean for five plants.
[b] VTP, vacuolar targeting peptide.

In contrast to the control plants, represented by untransformed wild-type plants and by plants transformed with an empty vector, all transformants with the tobacco genes encoding acidic chitinase (PR-3 [=PR-Q]) or acidic β-1,3-glucanases (PR-2 and PR-Q′) showed no change in the total chitinase or β-1,3-glucanase activities in their root systems (Table 2). In these transformants and in the plants transformed with the PR-1a, PR-4, and PR-5 genes, constitutive expression of the respective PRs in the roots was verified by immunoblot assays. Immunoblots with root extracts of the transgenic plants showed an intense band of the expected molecular size when examined with the appropriate antibodies, whereas control immunoblots with root extracts from untransformed plants or plants transformed with an empty vector showed no visible reaction (data not shown).

Colonization of transgenic plants by VAM fungi. The time courses of colonization of roots by VAM fungi for the control plants (empty-vector and wild-type plants) and for the transgenic plants expressing various PRs were compared (Table 3). With the exception of the transgenic plants expressing PR-2, the time course and extent of colonization were not significantly affected by the expression of any of the genes encoding PRs. The transformants expressing the PR-2 gene showed a significantly reduced extent of colonization from 4 weeks until the end of the experiment (Table 3).

The percentage of root length colonized 6 weeks after inoculation was reduced twofold in the plant expressing the PR-2 protein compared with that in the control. A random root sample, measuring 5 cm total, was taken from each of four plants per line and evaluated separately. The total length colonized in the control plant was 1.25 ± 0.36 cm (mean ± standard error of the mean), i.e., 25% ± 4%; the total length colonized in the plant expressing PR-2 was 0.50 ± 0.20 cm, i.e., 10% ± 4%.

Light microscopy showed differences in the fungal structures in the roots of the PR-2 transformant compared with those in the roots of control plants. Observation of individual regions of

colonization in control plants showed that the mycorrhizal structures were generally extended over a large area and arbuscules were abundant, and internal hyphae could be observed spreading throughout the whole root. In the PR-2 transformants, the mycorrhizal structures within a colonization unit were spatially more restricted, and there were comparatively fewer arbuscules than in the control plants. Hyphae in the plants expressing the PR-2 protein appeared to be thinner than hyphae in control plants.

DISCUSSION

The roots of the majority of herbaceous plants, including most crop plants, are colonized by beneficial symbiotic VAM fungi (13, 31). These endophytic fungi belong to the order *Glomales*; like most higher fungi, they have cell walls containing chitin and β-1,3-glucan (5), and they may be susceptible to chitinases and β-1,3-glucanases in a way similar to that of saprophytic and pathogenic ascomycetes and basidiomycetes (19, 29). During colonization of host plants by VAM fungi, these two antifungal hydrolases were initially induced but later suppressed in the colonized roots (16, 32, 37). Chitinase and β-1,3-glucanase activities were increased also in the roots of some nonhost plants inoculated with VAM fungi (35). In view of these findings, it was of interest to determine the ability of VAM fungi to colonize transgenic plants expressing high levels of chitinases and β-1,3-glucanases in their root systems.

In previous work, we have already shown that transgenic *N. sylvestris* plants constitutively expressing different forms of chitinase are colonized to the same degree by *G. mosseae* as control plants lacking the chitinase transgene (36). Here, we extend these observations to tobacco plants constitutively expressing vacuolar, ethylene-regulated class I chitinase (20) as well as vacuolar, basic class I β-1,3-glucanase (20). As in the case of *N. sylvestris*, expression of these transgenes did not interfere with the establishment of VAM. Not only did the final level of colonization remain unchanged but the time courses of colonization were identical in transgenic and control plants. Of particular interest is the observation that plants expressing the two enzymes simultaneously are as susceptible as controls to VAM fungus colonization. Previous work with pathogens and saprophytes growing on synthetic media has demonstrated that combinations of these two enzymes are much more efficient in inhibiting fungal growth than either enzyme alone (19, 29), and coexpression of chitinase and glucanase in transgenic tobacco has given substantially greater protection against *C. nicotianae* than either transgene alone (42).

What is the reason for the failure of the constitutively expressed basic chitinases and β-1,3-glucanases to inhibit growth of the VAM fungi? One possibility is that these enzymes, which were highly expressed in the roots of the transgenic plants in this study, may not reach their potential targets in the cell walls of the VAM fungi. Although their localization in the roots of transgenic plants has not yet been studied, the enzymes are normally localized in the plant vacuole and may not come into contact with fungi growing in the intercellular space (2, 3, 36). However, chitinase lacking the vacuolar targeting peptide is expected to be secreted (23), and it is ineffective as well (Table 3) (36). In this respect, it is interesting that living intercellular mycelia of VAM fungi do not bind chitinase (32); it has therefore been proposed that the chitin-glucan layer of the cell wall may be protected by a cover of proteins or alkali-soluble polysaccharides (6). In addition, it has been observed that pathogenic fungi become adapted to the presence of chitinase and β-1,3-glucanase in their surroundings, perhaps by altering their cell wall structures and thereby protecting themselves (3). A similar process may occur in mutualistic fungi.

In addition to the basic class I chitinases and β-1,3-glucanases, there are acidic isoforms of these enzymes which are secreted into the intercellular space (20). Transgenic plants expressing the acidic class III cucumber chitinase, the acidic class II chitinase represented by PR-3 (=PR-Q), or the acidic class III tobacco β-1,3-glucanase represented by PR-Q′ were all colonized by VAM fungi to the same extent as were control lines. Similarly, colonization of tobacco by VAM fungi was not affected by expression of PR-1a, a protein with unknown enzymatic functions but which was shown to be effective in protecting transgenic plants against infection by two pathogenic oomycetes, *P. tabacina* and *Phytophthora parasitica* (1); of PR-4, a protein with no known enzymatic function and no reported antifungal potential (10); or of PR-5, a homolog of the sweet protein thaumatin and of osmotin which displays activity against the oomycete *Phytophthora infestans* (40).

A surprise, however, was the finding that plants expressing high levels of the acidic class II β-1,3-glucanase PR-2 had a reduced symbiotic potential. In the transgenic plants expressing PR-2, colonization by VAM fungi occurred more slowly and did not spread as well as it did in control plants. This type of β-1,3-glucanase had very little antifungal potential when assayed with various fungi on artificial growth media, either alone or in combination with other PRs (29). Whether this enzyme acts directly on the mycorrhizal fungus by altering its cell wall structure or whether it releases oligosaccharide signals from the fungal cell walls that act on the plant or on the fungus (3) is an interesting question.

There is considerable interest in using transgenic plants showing enhanced resistance against plant pathogens in agriculture, and the beneficial effect of such transgenes on plant health is emphasized (9, 15). Our results indicate, for the example of PR-2, that beneficial symbiotic fungi may be affected adversely by transgenes introduced into the plant. We therefore suggest the testing of transgenic plants not only with respect to disease resistance but also with respect to their symbiotic abilities.

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